

## Systemic acquired resistance induced by BTH in papaya

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### Abstract

Systemic acquired resistance (SAR) in *Carica papaya* L. is induced by benzothiadiazole (BTH). The response is manifested by increased tolerance to infection by the virulent pathogen *Phytophthora palmivora*, by increased  $\beta$ -1,3-glucanase and chitinase activities, and by increased accumulation of a PR1 mRNA. Infection of untreated papaya by *P. palmivora* also induced  $\beta$ -1,3-glucanase and chitinase activities but at much lower levels. This response to *P. palmivora* is characteristic of a compatible interaction. Papaya has at least four members of the PR-1 gene family; BTH reduces mRNA accumulation for two of these and increases it in the other two. One of these, PR-1d, is induced over 20-fold; mRNA accumulation for this gene increased for at least 14 days after BTH treatment. In contrast, both chitinase and  $\beta$ -1,3-glucanase activities peaked after 1–2 days then returned to base levels at approximately 10 days. Papaya has an NPR1 gene that contains structural domains conserved with arabidopsis; these domains are involved in protein–protein interactions and nuclear localization, which are essential for function in SAR of arabidopsis. The papaya NPR1 gene is expressed constitutively and is slightly induced by BTH treatment. Overall, these findings indicate the basic elements of papaya SAR resemble the pathway as described in arabidopsis and tobacco.

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**Keywords:** Benzothiadiazole;  $\beta$ -1,3-Glucanase; *Carica papaya*; Chitinase; NPR1; *Phytophthora palmivora*; PR1; Systemic acquired resistance

### 1. Introduction

Systemic acquired resistance (SAR) is an inducible defense response found in a large range of plant species. Common features of SAR include:

1. Enhanced systemic resistance against a broad spectrum of pathogens, which is induced by pre-inoculation with an avirulent pathogen;
2. Increased endogenous accumulation of salicylic acid (SA);
3. Inducible by exogenous application of SA or related molecules like benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) or 2,6-dichloroisonicotinic acid (INA);

4. Correlates, and is in part due to, the elevated expression of numerous defense-related genes, including pathogenesis-related (PR) genes;
5. Signal transduction and induction of PR genes regulated by NPR1 gene product.

These features have been characterized primarily in the model systems arabidopsis and tobacco. Other studied species share most or all of these features, but there are also important interspecific differences, including kinetics of PR gene induction, dose response and duration of SAR from inducing treatments like BTH, and the particular array of defense genes that are induced by a particular treatment.

Examples of interspecific differences include levels of endogenous SA and response to exogenously applied SA or related compounds. In potato, tomato, soybean, and rice [50], baseline levels of SA are far higher than those in arabidopsis or tobacco. Exogenous application of SA to potato does not further increase endogenous SA levels and does not induce resistance against *Phytophthora infestans*. It does, however, induce local accumulation of PR1 protein

**Abbreviations:** BTH, benzothiadiazole; INA, 2,6-dichloroisonicotinic acid; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance.

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[11], and the tobacco PR1a promoter is induced by BTH root drench in transgenic potato plants [49]. Treatment with the pathogen elicitor arachidonic acid does induce locally elevated SA in potato; however, SA levels in systemic leaves do not increase. Localized treatment with arachidonic acid induces systemic resistance to *P. infestans* and *Alternaria solani* [11]. This evidence argues against a role for SA in potato SAR; however, in *nahG* transgenic potatoes, which accumulate much reduced levels of SA, arachidonic acid does not induce resistance to *P. infestans*. One hypothesis to explain these results is that potato SAR involves elevated sensitivity to SA, not the production of elevated levels of SA as in arabidopsis and tobacco [50].

Other important interspecific differences are seen in the specific genes induced by different SAR-inducing treatments. In tobacco and arabidopsis, exogenous application of SA ‘induces resistance against the same pathogens and accumulation of the same set of RNAs as observed with pathogen infection’ [21]. In rice, however, the set of genes induced by the avirulent pathogen *Pseudomonas syringae* pv. *syringae* and those induced by BTH do not overlap [40]. Following *P. syringae* pv. *syringae* inoculation of rice, the activities of phenylalanine ammonia lyase, coniferyl alcohol dehydrogenase, peroxidase,  $\beta$ -1,3-glucanase, and chitinase increased locally but not systemically; however, systemic resistance against *Pyricularia oryzae* was induced [43]. Wheat shows complete non-host resistance to *Erysiphe graminis* f. sp. *hordei*. Pre-inoculation with this fungus [39] or BTH treatment [18] leads to enhanced protection against *E. graminis* f. sp. *tritici*, the causative agent of wheat powdery mildew. However, several tested PR genes that are induced by *E. graminis* are not induced by BTH [26,37].

Induction kinetics of PR genes vary between species. In many plants, BTH-induced PR gene expression appears to be transient. In arabidopsis, RNA levels of PR-1, PR-2, and PR-5 peak one day after treatment and return to basal levels approximately 5 days after treatment [21]. Maize PR-1 and PR-5 display similar kinetics, peaking 2–3 days after treatment and then decreasing [27]. However, in BTH-treated tobacco the elevated transcript levels of PR-1, PR-2, PR-3, PR-4, PR-5 and five other defense-related genes peak at approximately 3 days and do not significantly decline even after 20 days [17]. In wheat, transcripts of four defense-related genes peak 1–4 days after treatment and then decrease significantly [18].

Papaya is subject to a number of bacterial, fungal, and Oomycete diseases, including devastating root rots and blights caused by *Phytophthora palmivora* [31]. Management and manipulation of endogenous defense responses provide potentially attractive methods of protection against these diseases, especially as some existing control methods become less acceptable to consumers, or even outlawed. Whether papaya defense responses can be farmer or breeder controlled is not known; to understand pathogen defense responses in a less-studied species like papaya, a generalized scheme of SAR needs to be validated. To do so,

this work reports the effect of BTH treatment on PR enzyme activities, PR1 mRNA accumulation, and resistance to *P. palmivora*. Additionally, a papaya homolog of the arabidopsis NPR1 gene, a key SAR regulatory gene [6], was isolated and the deduced peptide sequence analyzed with regard to the domains required for function in arabidopsis.

## 2. Materials and methods

### 2.1. Plant growth

*Carica papaya* L. cv. SunUp or cv. Kapoho seeds were germinated in the greenhouse in flats containing potting soil. Three weeks after germination, when seedlings reached approximately 2 cm in height, they were transplanted individually into 4-in. pots containing the same potting soil. Four-week-old SunUp seedlings were divided into four groups of 60 plants each to serve as experimental replicates for disease resistance and enzyme activity experiments. Each replicate contained 12 treatment blocks of five plants each, randomized in a complete block design. Plants were grown in the greenhouse at Aiea, Hawaii, during the winter with temperatures of 20–26 °C and daylight of approximately 12 h. Three-month-old Kapoho plants were used for RNA isolation.

### 2.2. BTH treatment and pathogen inoculation

Test solutions were applied to plants by root drench application. BTH (Novartis Crop Protection) was applied as a suspension of the formulated wettable powder (50% active ingredient) at concentrations of 0, 1, 5, 25, and 100  $\mu$ M in distilled H<sub>2</sub>O. For pathogen challenge experiments,  $1 \times 10^4$  *P. palmivora* zoospores in H<sub>2</sub>O were applied by root drench 1 week after BTH or H<sub>2</sub>O treatment. One treatment block of inoculated plants was treated with 100 ppm Ridomil® as a fungicide control. Plants were maintained under identical conditions in the greenhouse for 6 weeks and then evaluated for response to disease symptoms and BTH treatment.

### 2.3. Symptom evaluation

Effects of chemical treatments without pathogen inoculation were evaluated by measuring plant height and stem diameter biweekly up to the time that disease response was determined on the inoculated plants. Disease symptoms were scored 6 weeks after pathogen inoculation on a scale of 0, symptomless; 1, slight leaf yellowing; 2, leaf yellowing and slight wilting; 3, leaf wilt and collapse; 4, leaf abscission and stem wilt; and 5, dead.

### 2.4. Protein extraction

Leaves and roots were harvested at various times after treatment. Samples were stored at –80 °C until analysis.

Three replicate extractions were performed for each sample. Frozen leaves (3 g) were ground under liquid nitrogen in 6 ml of 0.1 M phosphate buffer at pH 7, containing phenylmethylsulfonyl fluoride (PMSF) (1.0 mM) and 2,2'-dithiopyrindine (1.5 mM). The homogenates were filtered (Whatman no. 1) and centrifuged at 13,800g for 20 min at 4 °C. Supernatants were used for enzyme assays.

## 2.5. Chitinase activity assay

Chitinase activity in the crude protein extract was determined by a colorimetric assay [34]. Specifically, chitin powder (Sigma) was washed three times with 0.1 M sodium acetate buffer (pH 5.2) to remove colored materials that would interfere with the enzyme assay. The reaction mixture contained 0.5 mg of washed chitin to which were added 50 µl of crude enzyme extract and made up to a final volume of 0.5 ml with 0.1 M sodium acetate buffer (pH 5.2). The mixture was incubated in a shaking water bath at 37 °C for 1 h then centrifuged at 12,000g for 30 min to remove the chitin substrate. After centrifugation, 0.3 ml of the supernatant was placed in a 4 ml reaction tube and incubated at 37 °C for 1 h with 5 µl of 25% β-glucuronidase to hydrolyze the chitin oligomer. The amount of *N*-acetylglucosamine (Glc-Nac) produced in the reaction was determined by adding 0.1 ml of 0.6 M potassium tetraborate and heating the reaction mixture for 3 min in a boiling water bath. After cooling on ice, 1 ml of the color reagent diluted 1:2 with glacial acetic acid was added. The color reagent stock solution contained 10% 4-(dimethylamino)-benzaldehyde in 87.5 ml of glacial acetic acid and 12.5 ml of 11.5 M HCl. The samples were cooled and  $A_{585}$  was determined within 10 min with a DU-70 spectrophotometer (Beckman). Three concentrations of Glc-Nac (0.1, 0.2 and 0.4 µM) were used to produce a standard curve. Enzyme activity is reported in katals (kat), defined as the amount of activity required to catalyze the formation of 1 mol of Glc-Nac s<sup>-1</sup>.

## 2.6. β-1,3-Glucanase activity assay

β-1,3-Glucanase activity in the crude protein extracts was assayed by measuring the rate of production for reducing sugars using laminarin as a substrate. Reducing sugars were assayed as described [28]. The reaction mixture containing the crude enzyme extract and the laminarin substrate was incubated at 37 °C and added to an equal volume of the Nelson alkaline copper reagent. Glucose was used as a standard. Enzyme activity is reported in katals (kat) defined as the amount of activity required to catalyze the formation of 1 mol of glucose equivalents s<sup>-1</sup>.

## 2.7. RNA isolation

RNA was extracted from 3-month-old *C. papaya* cv Kapoho seedlings grown and treated with BTH (root drench)

24 h before harvest. For northern blots, small scale RNA isolations were performed with 100 mg leaf tissue, ground to a fine powder under liquid N<sub>2</sub>, and extracted as described [4]. For cDNA synthesis, large scale isolations were performed with 30 g leaf tissue, ground to a fine powder under liquid N<sub>2</sub>, and extracted by the acid guanidinium thiocyanate–phenol–chloroform method [9]. RNA concentration was estimated using the RiboGreen RNA Quantitation kit (Molecular Probes) and a Fluoro-lite1000 (Dynex Technologies) fluorescence plate reader.

## 2.8. Cloning of papaya PR1 and NPR1 genes by RT-PCR

First strand cDNA was synthesized from 2 µg total RNA using the 3' RACE kit for rapid amplification of cDNA ends (Invitrogen) according to the manufacturer's protocol. One microliter of the resulting cDNA was used without further purification as template for subsequent PCR amplification. Partial PR1 cDNAs were obtained by two rounds of nested PCR with an adapter primer (AUAP, Invitrogen) complementary to the oligo-dT adapter primer used to prime first strand cDNA synthesis and a (degenerate) gene specific primer. PCRs contained 1 × reaction buffer (Promega), 4 mM MgCl<sub>2</sub>, 2.5 µM each primer, 200 µM each dNTP, and 2.5 U *Taq* polymerase (Promega) in a 50 µl reaction volume. Amplification was for 35 cycles of 30 s each at 94, 54, and 72 °C. Products were separated on a 1% agarose gel; a band of the expected size was excised and purified by GeneClean (Q-Biogene) according to the manufacturer's protocol. Approximately 1 ng of the purified product was used as template for the second round PCR, which contained the same reaction mix as above, amplified for 35 cycles of 30 s each at 94, 56, and 72 °C. PR1 gene specific primers were made to the conserved peptide GHYTQVW [48]: SPPR1 (5' GWR TGY SGW CAC TAY ACT CA 3'), used for first round amplification, and SPPR2 (5' TGY SGW CAC TAY ACT CAG RT 3'), used for the second round. The AUAP adapter primer (5' GGC CAC GCG TCG ACT AGT AC 3') was used for both rounds of amplification. Products from second round amplification were blunt-end cloned in pPCR-Script (Stratagene). PR1 partial cDNA sequences were deposited as GenBank accession numbers CF569398 (a), CF569399 (b), CF569400 (c), and CF569397 (d).

A partial cDNA clone for papaya NPR1 was obtained by RT-PCR using degenerate primers based on a region conserved in the arabidopsis, tobacco and tomato NPR1 proteins: F2 (5' CTK CAT GTT GCW GCS ATG 3') is in the ankyrin repeat region (from arabidopsis amino acid 332 to 342), and R2 (5' AAG AAB CGT TTY CCR AGT TC 3') is downstream of the ankyrin repeats (from arabidopsis amino acid 500 to 512). The PCR mixture contained 1 × *Taq* reaction buffer (Promega), 4 mM MgCl<sub>2</sub>, 1.25 µM each primer, 200 µM each dNTP, and 2.5 U *Taq* polymerase (Promega) in a 50 µl reaction volume. Amplification was for 32 cycles of 94 °C, 30 s;

54 °C, 30 s, 72 °C, 40 s. The resulting product was cloned in pPCR-Script (Stratagene). Sequence of this clone was used to design two new gene specific primers (5' AGA ATC CAA GAG GAG CAC CT 3' and 5' ACT CTA ACC GCG AGC AGA AC 3'), which were used to obtain the downstream portion of the cDNA by 3' RACE. After obtaining partial CpNPR1 genomic sequence, another 3' RACE was carried out with cv. Sun-Up mRNA and the gene specific primer (5' CAA CTA ACA CAG ACA CGC TT 3'). Amplification was with 1.5 U *Pfu* DNA polymerase (Promega) in 1 × *Pfu* reaction buffer (Promega), 200 μM each dNTP, 0.5 μM each primer in a volume of 50 μl for 35 cycles of 95 °C, 30 s; 56 °C, 30 s; and 72 °C, 4 min. The product was blunt-end cloned in pPCR-Script (Stratagene). Sequence of the partial CpNPR1 cDNAs were deposited as GenBank accession numbers CF588412 and AY548108.

## 2.9. RNA gel-blot hybridizations

Ten micrograms total RNA per sample was separated on a 1.5% agarose formaldehyde denaturing gel, then transferred to Hybond N+ membranes (Amersham) by capillary transfer as described [44]. Hybridization and stringency washes were performed as described [10]. DNA probes were <sup>32</sup>P labeled by the random priming method [16]. Washed blots were exposed to storage phosphor screens and read on a GS505 Molecular Imaging System (Bio-Rad).

## 2.10. Quantitative RT-PCR

Total RNA was extracted from leaves of five 3-month-old seedlings 3 days after BTH or H<sub>2</sub>O treatments. RNA was treated with RQ1 DNase (Promega) per manufacturer's protocol to remove genomic DNA contamination. Two micrograms of treated total RNA was used for first strand cDNA synthesis in a 100 μl reaction using the Taqman reverse transcription reagents kit (Applied Biosystems) per manufacturer's protocol. The resulting cDNA was diluted with 450 μl H<sub>2</sub>O, and 8 μl of this 5.5 × dilution was used as template per 50 μl PCR. PCR was performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen) with 0.6 μM each primer on a 7900HT Sequence Detection System (Applied Biosystems). Cycling profile was 50 °C, 2 min; 95 °C, 10 min; 40 cycles of 95 °C, 15 s; 58 °C, 20 s; 72 °C, 30 s; followed by denaturation for melting curve analysis. Each sample was replicated twice and normalized to actin. Analysis of qPCR data was by the 'Comparative C<sub>T</sub>' method as described [2]. Briefly, for each gene, ΔC<sub>T</sub> was derived by subtracting C<sub>T</sub> H<sub>2</sub>O treatment from C<sub>T</sub> BTH treatment. ΔΔC<sub>T</sub> was derived by subtracting ΔC<sub>T</sub> actin from ΔC<sub>T</sub> of the gene being analyzed. Induction, normalized to actin, was determined by evaluating 2<sup>−ΔΔC<sub>T</sub></sup>.

Primers used were CPNPR1F, TTCCTGGTGGAGGTGCTCTA; CPNPR1R, CATAACCTGCCGAGCAATC; CPPR1AF, CCCGGAGGATCATAGTTACAAG;

CPPR1AR, ACTCAGGTTGTGTGGAGAAC; CPPR1BF, TGCGGTCACTATACTCAGGT; CPPR1BR, GATAGTTCCGCCATTGTTGC; CPPR1CF, AATTGGGTGCGCGAGAGT; CPPR1CR, GGCCTTTCGCCAATGTAATTCC; CPPR1DF, GCGGTCACTATACTCAGGTT; CPPR1DR, TGCCTGGAGGATCATAGTTG. Primers were designed with Primer Designer 5 (Scientific and Educational Software).

## 2.11. Isolation of papaya NPR1 gene from BAC library

Two BAC filters covering approximately 13.7 × papaya-genome equivalents [25] were prehybridized in 100 ml hybridization buffer at 65 °C. After 4 h the prehybridization buffer was removed and 100 ml fresh hybridization buffer was added with the <sup>32</sup>P dCTP-labeled papaya NPR1 cDNA and hybridized for 48 h at 65 °C. Hybridization and stringency washes were as described [10]. After washing, filters were exposed to Blue Sensitive Autoradiographic film (Marsh Bio Products, Inc.) at −80 °C. Positive BAC clones were identified by BAC-DMS 2.1 software [14]. Two subclones of the BAC were sequenced (GenBank accession number AY550242).

## 2.12. Data analyses

After angular transformation, the data for disease rating, plant height, and stem diameter were independently subjected to one-way analysis of variance (ANOVA) using SAS (Statistical Analysis System Inc., Cary, NC, USA), followed by Waller-Duncan *K*-ratio *T* test. Chitinase and β-1,3-glucanase activities were compared as the means ± 1 SD of three replicates. Sequence comparisons were performed with Align Plus 5 [41]. qRT-PCR data for mRNA levels were compared as the means ± 1 SD of two replicates.

# 3. Results

## 3.1. Effect of BTH on plant growth

Four-week-old papaya 'SunUp' seedlings were treated with various BTH concentrations without subsequent pathogen inoculation to determine the effect of BTH on papaya plant growth. Plant heights and diameters were measured 6 weeks after treatment. No significant effect was observed in either plant height or stem diameter of seedlings treated with up to 25 μM BTH. Treatment with 100 μM BTH or higher caused a transitory loss of chlorophyll in seedlings 2 weeks after application; however, the seedlings recovered at 6 weeks with only slight reductions in plant height and stem diameter (Table 1) and were fully recovered with no observable effect on plant growth at 10 weeks post treatment (data not shown).



Table 1

The effect of a single BTH drench treatment on *Phytophthora* root rot symptoms and growth in SunUp papaya plants

Treatment	Disease rating <sup>a</sup>	Plant heights <sup>b</sup> (cm)	Stem diameter <sup>b</sup> (mm)
H <sub>2</sub> O	3.40a	90.1a	17.1a
BTH at 1.0 $\mu$ M	3.25a	88.1a	16.0a
BTH at 5.0 $\mu$ M	0.95b	91.0a	16.0a
BTH at 25.0 $\mu$ M	0.64bc	88.4a	16.4a
BTH at 100 $\mu$ M	0.60bc	85.2b	14.5b
Ridomil	0.1c	—	—

<sup>a</sup> Disease rating was assessed 6-weeks after *P. palmivora* inoculation, scored on a scale of 0 (healthy) to 5 (dead plants).

<sup>b</sup> The growth data were collected six weeks after treatment without *P. palmivora* inoculation. Letters a–c are class measures of the Waller-Duncan *K*-ratio *T* test. Means with the same letter are not significantly different at  $p < 0.05$ .

### 3.2. Effect of BTH treatment on severity of *P. palmivora* disease in papaya seedlings

Untreated SunUp plants were very susceptible to *P. palmivora* inoculation: within 2 weeks after inoculation, the control plants started to develop symptoms typically associated with root rot including yellowing and wilting of leaves. When papaya plants were treated with BTH concentrations ranging from 0 to 100  $\mu$ M and subsequently challenged by *P. palmivora* zoospore inoculation, these symptoms were reduced in direct proportion to the concentration of BTH used in the pre-treatment (Table 1). Plants pre-treated with 25 or 100  $\mu$ M BTH expressed very minor root rot disease symptoms consisting of minor leaf yellowing. On a comparative basis, symptom development in these treatment groups was reduced to less than 20% of the levels exhibited by water-treated controls where the *P. palmivora* caused defoliation, stunted growth, and wilting of the seedlings. Seedlings treated with 5–12.5  $\mu$ M BTH expressed an intermediate level of root rot disease symptoms consisting of considerable leaf yellowing that

was about 30% of the level of symptoms developed in the water controls. The fungicide Ridomil<sup>®</sup>, in comparison, completely suppressed visible symptoms of *P. palmivora* disease. Disease ratings in all BTH treatment groups except 1  $\mu$ M were significantly different from the water control plants. *P. palmivora* challenge experiments were repeated three times during different seasons of the year and similar results were obtained for all three experiments.

To confirm the presence of *P. palmivora* in the diseased plants, roots of diseased papaya plants were washed, surface sterilized, and placed on V8 juice culture medium. *P. palmivora* was re-isolated from all diseased plants tested.

Roots of papaya plants were also visually evaluated 6 weeks after *P. palmivora* inoculation (representative plants are shown in Fig. 1). Water control plants inoculated with *P. palmivora* showed symptoms typical of root rot disease. Plants pretreated with BTH at 100 and 500  $\mu$ M prior to *P. palmivora* inoculation did not show root rot symptoms.

### 3.3. Differential systemic induction of PR proteins by *P. palmivora* and BTH treatment

$\beta$ -1,3-Glucanase activities were significantly higher in leaves than roots in all treatment groups including water control, *P. palmivora* inoculation, and 100  $\mu$ M BTH treatment (Fig. 2). In these experiments BTH treatments were applied to roots, not leaves, so effects observed in the roots may be local; however, leaf effects are systemic. In roots,  $\beta$ -1,3-glucanase activity was increased twofold by BTH but not increased by *P. palmivora* inoculation. In leaves,  $\beta$ -1,3-glucanase activity was increased threefold by BTH treatment but less than twofold by *P. palmivora* root inoculation.

Like  $\beta$ -1,3-glucanase, chitinase activity was higher in leaves than in roots for all treatments (Fig. 3). Chitinase activity in leaves treated (root drench) by BTH were sixfold higher than the water control, but less than twofold higher in *P. palmivora* inoculated plants. In roots, BTH increased

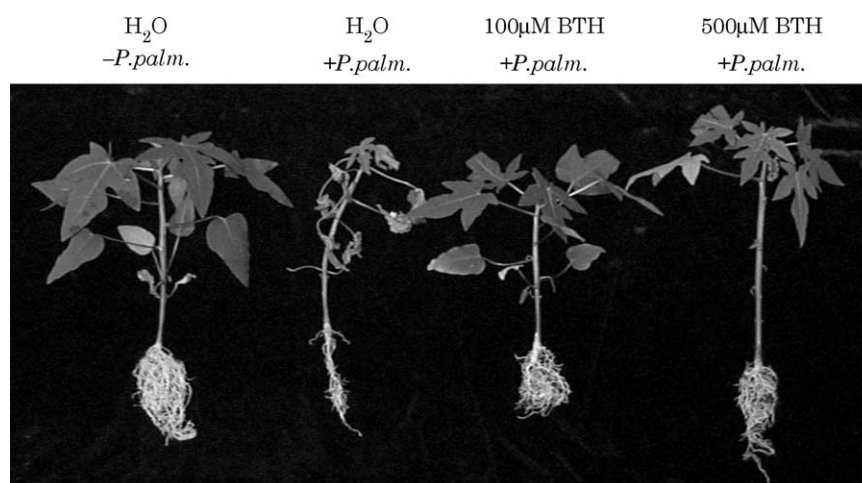


Fig. 1. Effect of BTH on papaya *Phytophthora* root rot.

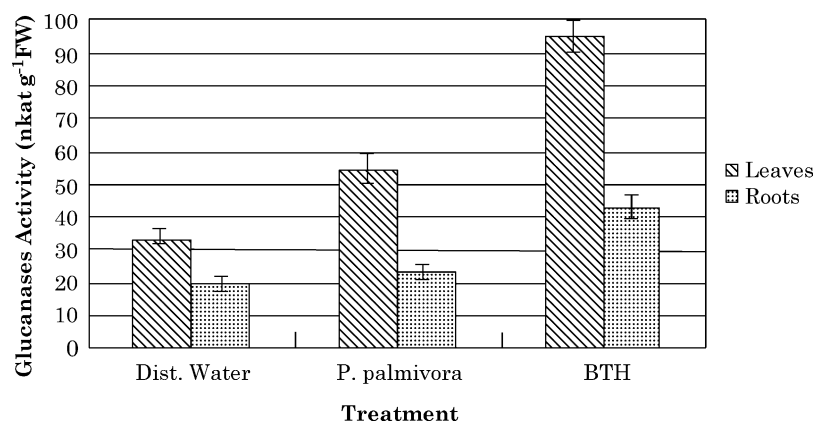


Fig. 2.  $\beta$ -1,3-Glucanase activity 24 h after root drench of 4-week-old SunUp papaya seedlings with 5000 zoospores of *P. palmivora* and 100  $\mu$ M BTH.

chitinase activity twofold, but *P. palmivora* inoculation produced no increase.

### 3.4. PR enzyme activity kinetics following BTH treatment

Activities of  $\beta$ -1,3-glucanase and chitinase were increased within 2 h of 100  $\mu$ M BTH treatment and reached a maximum at 1 day ( $\beta$ -1,3-glucanase) and 2 days (chitinase) post-treatment. The elevated activities lasted up to 10 days before returning to base levels (Fig. 4).

### 3.5. Four PR-1 genes in papaya

Nested degenerate primers were designed based on the peptide GHYTQVW, which is highly conserved among all PR-1 proteins [48]. These primers were used to isolate partial cDNAs from four distinct PR1 genes. The PR1 cDNA clones include 135 nucleotides in the coding region and 122–145 nucleotides in the 3' untranslated region (3' UTR). These cDNAs encode the fourth  $\alpha$ -helix ( $\alpha$  IV) and the third and fourth  $\beta$ -strands ( $\beta$ C and  $\beta$ D) of the PR-1 protein [48].

The four PR1 homologues were designated PR-1a–d. CpPR-1a and CpPR-1c show the highest identity (over the region sequenced) among the four, while CpPR-1d shows

the highest identity to the extensively characterized tobacco PR-1a (Fig. 5). Relative to tobacco PR-1a and the other three CpPR-1 genes, CpPR-1b has a short insertion immediately 5' of the stop codon; this could indicate the presence of a vacuole targeting signal. mRNA for PR-1a in leaves decreased approximately fivefold 3 days after root drench with BTH, PR-1b increased significantly although variation in measurements of this mRNA were quite large, and PR-1c decreased approximately threefold. In contrast, PR-1d increased over 20-fold (Table 2). Already at 6 h after BTH root drench, PR-1d mRNA showed a significant increase in leaves (Fig. 6A). Papaya PR-1d mRNA levels continued increasing for 14 days (Fig. 6B).

### 3.6. NPR1

A partial cDNA containing part of the ankyrin repeat region for papaya NPR1 was produced by RT-PCR. Sequence from this clone was used to isolate the downstream fragment of the gene by 3' RACE; the upstream fragment of the gene was obtained from a BAC clone [25].

The papaya NPR1 deduced peptide sequence has 71.04% similarity with rice and 66.84% similarity with arabidopsis NPR1 proteins (Fig. 7). A similar level of homology was reported between the arabidopsis NPR1 protein

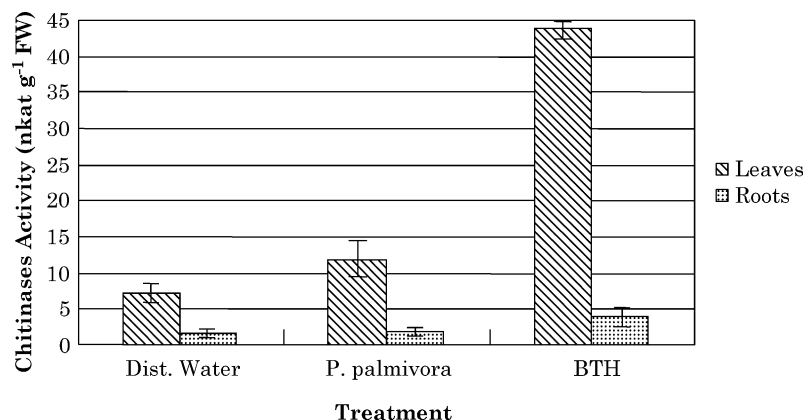


Fig. 3. Chitinase activity 24 h after root drench of 4-week-old SunUp papaya seedlings with 5000 zoospores of *P. palmivora* and 100  $\mu$ M BTH.

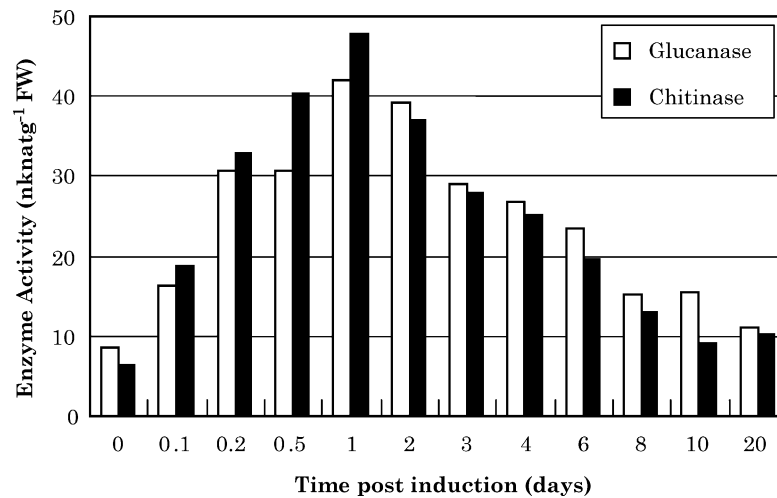


Fig. 4. Time course of  $\beta$ -1,3-glucanase and chitinase activities in roots of 3-week-old SunUp papaya seedlings following 100  $\mu$ M BTH root drench.

and homologs from tobacco and tomato [7]. We have identified four exons and three introns in the papaya NPR1 gene, with the position of the introns identical to that of the arabidopsis gene.

As in arabidopsis, exon 2 of papaya NPR1 encodes four ankyrin repeats (amino acids 268–396). This ankyrin repeat region shows 64.3% identity and 72.9% similarity to the corresponding region of the arabidopsis protein, slightly higher than the average homology between the entire proteins. Exon 1 of papaya NPR1 contains a predicted poxvirus and zinc finger (POZ) domain [3], which is also present in the arabidopsis protein. Over this domain, both arabidopsis and papaya match 28 of 33 (85%) consensus amino acids [3]. Arabidopsis NPR1 contains a nuclear localization signal (NLS); four of the five amino acids required for nuclear localization of arabidopsis NPR1 are conserved in papaya, and also in rice [36] and tobacco [24].

Papaya NPR1 mRNA level is slightly elevated (approximately  $1.7 \times$ ) following BTH treatment (Table 2), which is comparable to induction of NPR1 seen in arabidopsis [6].

#### 4. Discussion

Papaya has an SAR response that is in many ways like that characterized in arabidopsis. BTH induces this response

and can be used as a convenient research tool and may provide a viable crop protection option.

BTH treatment of papaya induces increased chitinase and  $\beta$ -1,3-glucanase activities and increased accumulation of mRNA from two PR-1 genes, with little or no phytotoxic effect. The dose response is intermediate to that reported for arabidopsis [21] and tobacco [17]. BTH treatment as low as 5  $\mu$ M induces significantly enhanced tolerance to the virulent pathogen *P. palmivora*, which is not affected by addition of BTH (at up to 1.5 mM) to culture media (data not shown).

PR proteins were first described in tobacco plants exhibiting hypersensitive response to Tobacco Mosaic Virus. These leaves contained proteins not found in leaves of uninfected tobacco, and the proteins were named 'pathogenesis-related' (PR) proteins. The first four observed PR proteins were relatively stable at low pH, remained soluble even at pH 3, and were found predominantly in the extracellular space. Additional PR proteins discovered later included many which had basic pI values and which accumulated in the vacuole. This led to classification of PR proteins as acidic or basic isoforms, however, these classifications have not been shown to correlate well with differences in function, induction, or even compartmentation [23]. Although PR1 was among the first PR proteins to be identified, its function remains unknown. PR-1 proteins

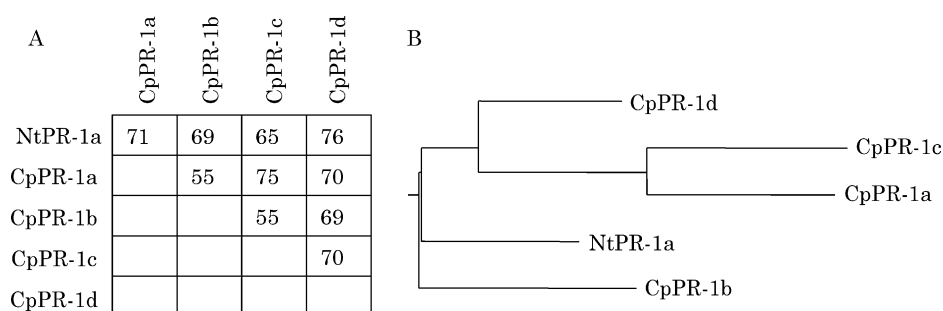


Fig. 5. Similarity of papaya PR-1 genes. (A) Percent identity with *N. tabacum* PR-1a (accession X06930). (B) Multiway alignment dendrogram.

Table 2  
Induction of NPR1 and PR-1 mRNA by BTH

Gene	BTH $C_T$	H <sub>2</sub> O $C_T$	$\Delta C_T$	$\Delta\Delta C_T$	Norm. induct.
Actin	23.35 ± 0.08	23.39 ± 0.08	−0.05 ± 0.11	0.00 ± 0.11	0.92–1.08
CpNPR1	31.86 ± 0.17	32.67 ± 0.13	−0.81 ± 0.22	−0.76 ± 0.22	1.46–1.97
CpPR1a	26.31 ± 0.01	23.92 ± 0.07	2.39 ± 0.07	2.44 ± 0.07	0.18–0.19
CpPR1b	33.89 ± 0.04	37.18 ± 1.68	−3.29 ± 1.68	−3.25 ± 1.68	2.96–30.34
CpPR1c	29.06 ± 0.01	27.71 ± 0.16	1.35 ± 0.16	1.39 ± 0.16	0.34–0.43
CpPR1d	29.93 ± 0.05	34.38 ± 0.21	−4.44 ± 0.22	−4.40 ± 0.22	18.13–24.49

Real-time RT-PCR quantification of mRNA species in leaves 3 days after BTH or H<sub>2</sub>O root drench treatment.  $C_T$ , threshold cycle;  $\Delta C_T$ , average BTH  $C_T$  minus average H<sub>2</sub>O  $C_T$ ;  $\Delta\Delta C_T$ ,  $\Delta C_T$  gene minus  $\Delta C_T$  actin; norm. induct., fold-increase in BTH treatment, normalized to actin =  $2^{-\Delta\Delta C_T}$ . Values are the mean from two PCRs ± 1.0 SD.

are encoded by a multigene family; some PR1 genes are induced by avirulent pathogen challenge or BTH treatment, and these induced PR1 genes are commonly used as a marker for SAR in both dicots, including arabidopsis and tobacco, and monocots, such as maize [17,18,21]. Other PR1 genes have been shown to be induced by jasmonic acid (JA), abscisic acid, kinetin, or protein phosphatase inhibitors [1,8,32,33,53]. While either SA and/or JA may act to induce some PR-1 genes, there is also evidence for an antagonistic effect between the two inducers, so that some basic PR-1 genes when activated by one of these inducers are repressed by simultaneous application of the other inducer [1,30]. Analysis of the tobacco PR-1a promoter has led to the identification of as-1 (activation sequence 1)—like *cis* elements which are required for induction by SA [19,46,47]. This *cis* element acts as a binding site for nuclear as-1-binding factor (ASF-1) and cellular salicylic acid response protein (SARP). The bZIP transcription factor TGA2.2 (also other members of the TGA family) can act as a major component of either of these complexes [29]. In an arabidopsis PR1 promoter a bZIP transcription factor binding site was also found to be required for INA induction; additionally a negative regulatory element was identified [22].

Papaya has a PR-1 gene family with at least four expressed members, CpPR-1a–d. While mRNA for all four was detectable in BTH-treated seedlings, CpPR-1a and CpPR-1c were down-regulated by the treatment, CpPR-1b was moderately up-regulated, and CpPR-1d, was up-regulated more than 20-fold. Of the four gene family, the two BTH down-regulated CpPR-1 genes are most similar to each other. PR-1 genes that do not respond to SA treatment [26,45] have been reported; however, to our knowledge, this is the first report of PR-1 genes that are down-regulated by treatment with BTH (or other analog of SA) in the absence of JA; i.e. antagonistic effects of SA and JA are seen when both, not one alone, are applied. However, other (non-PR-1) plant defense-related genes which are down-regulated by SA treatment alone are known [38], so it is not unexpected that some PR-1 genes might also show this pattern of regulation. The effect of JA or JA with SA on members of the CpPR-1 gene family is not yet known; however,

it appears that CpPR-1d can serve as a convenient marker of BTH induced SAR in papaya.

In arabidopsis, PR-1 mRNA accumulation peaks one day after BTH treatment and declines starting on day 2, returning to base levels approximately 5 days after treatment [21]. In BTH-treated tobacco the elevated transcript levels of PR-1 peak at approximately 3 days and do not significantly decline even after 20 days [17]. Papaya PR-1d mRNA increases steadily after BTH treatment, with no decrease through 14 days (Fig. 5A and B).

NPR1 plays an essential role in pathogen response signal transduction in arabidopsis; the null mutant from which the gene name is derived is a ‘nonexpresser of PR genes’ [5]. Homologs of the arabidopsis NPR1 gene have now been isolated from numerous other plant species [7,24,36], but functional analysis has been carried out primarily in arabidopsis. That analysis shows that NPR1 is a constitutively expressed (but see below) cytoplasmic protein [6,35] that is localized to the nucleus upon treatment with SA or related compounds [20]. In the nucleus, NPR1 interacts with some members of the TGA/OBF family of basic leucine zipper (bZIP) transcription factors [13,15,51,52]. Recently it was shown that SA reduces a disulfide bridge on TGA1, which increases TGA affinity for NPR1. When bound to NPR1, binding affinity of TGA1 for its cognate DNA sequence (as-1-like) is increased significantly [12]. TGA transcription factors bind as-1-like elements present in

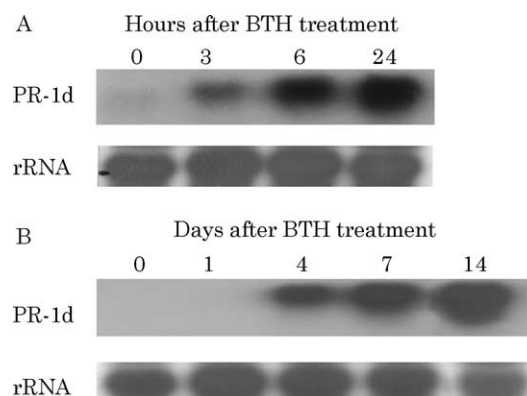


Fig. 6. Accumulation of PR-1d mRNA in leaves (A) following 10  $\mu$ M BTH root drench, 6-h exposure. (B) One-hour autoradiograph exposure.



	1				50
CpNPR1	MDYRTGTSDS	NDISNNSSTC	CVATN..TDT	LSHPLE...P	LTTPEISGLQ
OsNPR1		MEPPT	SHVTNAFSDS	DSASVEEGGA	DADADVEALR
AtNPR1	MDTTIDG	FADSYEISST	SFVATDNTDS	SIVYLAAEQV	LTGPDVSALQ
CpNPR1	LLSRNLLTIF	DS.SDFDFFS	DARLML....	GSGREIPVHR	CILSSRSPPFF
OsNPR1	RLSDNLAAAF	RSPEDFAFLA	DARIAVPGGG	GGGGDLLVHR	CVLSARSPFL
AtNPR1	LLSNSFESVF	DSPDD..FYS	DAKLVL....	SDGREVSFHR	CVLSARSSFF
CpNPR1	<u>KAIF.....</u>	<u>.SGSAFKERT</u>	<u>AKFRLKELAG</u>	<u>...DYDVG</u>	<u>DALVAVLAYL</u>
OsNPR1	<u>RGVFARRAAA</u>	<u>AAGGGGEDGG</u>	<u>ERLELRELLG</u>	<u>GGGEEVEVGY</u>	<u>EALRLVLDYL</u>
AtNPR1	<u>KSALA..AAK</u>	<u>KEKDSNNTAA</u>	<u>VKLELKEIA.</u>	<u>...KDYEVEGF</u>	<u>DSVVTVLAYV</u>
CpNPR1	YTGKVVWLPK	GVCVCVDEEC	SHVGCRPAVD	FLVEVLYVAF	TFQISELVAL
OsNPR1	YSGRVGDLPK	AACLCVDEDC	AHVGCHPAVA	FMAQVLFAS	TFQVAELTNL
AtNPR1	YSSRVRPFPK	GVSECADENC	CHVACRPAVD	FMLEVLYLAF	IFKIPELITL
CpNPR1	YQRHLLDIID	KVETDNILVI	LSVANICGKV	CDRLLGRCMD	IIVKSDVDAV
OsNPR1	FQRRLLDVLD	KVEVDNLLLI	LSVANLCNKS	CMKLLERCLD	MVVRSLNDMI
AtNPR1	YQRHLLDVVD	KVVIEDTLVI	LKLANICGKA	CMKLLDRCKE	IIVKSNVDMV
CpNPR1	TLDKSLPLSI	VKQIMDLRAE	CDTQGPEGRS	FPDKHKVRIH	RALDSDDEL
OsNPR1	TLEKSLPPDV	IKQIIDARLS	LGLISPENKG	FPNKHVRIH	RALDSDDEL
AtNPR1	SLEKSLPEEL	VKEIIDRRKE	LGLEVPKVK.	...KHVSNVH	KALDSDDEL
CpNPR1	<u>VRMLLKEART</u>	<u>NLDDAHALHY</u>	<u>AVAYCDAKTT</u>	<u>IELLDLGLAD</u>	<u>VNHRNSRGYT</u>
OsNPR1	<u>VRMLLLEGQT</u>	<u>NLDDAFALHY</u>	<u>AVEHCDSKIT</u>	<u>TELLDLALAD</u>	<u>VNHRNPRGYT</u>
AtNPR1	<u>VKLLLKEDHT</u>	<u>NLDDACALHF</u>	<u>AVAYCNVKA</u>	<u>TDLLKLDLAD</u>	<u>VNHRNPRGYT</u>
CpNPR1	<u>VLHIAAMRKE</u>	<u>PKLIVSLLTK</u>	<u>GARPSDLTPD</u>	<u>GRKALQISKR</u>	<u>LTKAADYYNT</u>
OsNPR1	<u>VLHIAARRRE</u>	<u>PKIIVSLLTK</u>	<u>GARPADVTFD</u>	<u>GRKAVQISKR</u>	<u>LTKQGDYFGV</u>
AtNPR1	<u>VLHVAAMRKE</u>	<u>PQLILSLLEK</u>	<u>GASASEATLE</u>	<u>GRTALMIKQ</u>	<u>ATMAVECNNI</u>
CpNPR1	<u>TEEGKAAPKD</u>	<u>RLCVEILEQA</u>	<u>ERRDPLLGEA</u>	<u>SLSLAKAGDD</u>	<u>FRMKLLYLEN</u>
OsNPR1	<u>TEEGKPSPKD</u>	<u>RLCIEILEQA</u>	<u>ERRDPLQGEA</u>	<u>SVSLAMAGES</u>	<u>LRGRLLYLEN</u>
AtNPR1	<u>PEQCKHSLKG</u>	<u>RLCVEILEQE</u>	<u>DKREQIPRDV</u>	<u>PPSFAVAAD</u>	<u>LKMTLLDLEN</u>
CpNPR1	RVGLAKLLFP	MEAKVAMDIA	QVNGTSEFTF	.D.GIN..SN	REQNTMDLNE
OsNPR1	RVALARIMFP	MEARVAMDIA	QVDGTLEFNL	GS.GANPPPE	RQRTTVDLNE
AtNPR1	RVALAQRLFP	TEAQAAMEIA	EMKGTCEFIV	TSLEPDRLTG	TKRTSPGVKI
CpNPR1	APFRIQEEHL	NRLRALSRTV	ELGKRFFPRC	SEVLNKMIDA	DDLSSLARLE
OsNPR1	SPFIMKEEHL	ARMTALSRTV	ELGKRFFPRC	SNVLDKIMD.	DETDVPS.LG
AtNPR1	APFRILEEHQ	SRLKALSRTV	ELGKRFFPRC	SAVLDQIMNC	EDLTQLACGE
CpNPR1	<u>HDTPEERRLK</u>	<u>KRRYMELQDI</u>	<u>LSKAFSEDKE</u>	<u>EFDKSTF..S</u>	<u>SSSSSKSVGP</u>
OsNPR1	<u>RDTSAE...K</u>	<u>RKRFDLQDV</u>	<u>LQKAFHEDKE</u>	<u>ENDRSL..S</u>	<u>SSSSSTSIGA</u>
AtNPR1	<u>DDTAEKRLQK</u>	<u>KQRYMEIQET</u>	<u>LKKAFSEDNL</u>	<u>ELGNSSLTDS</u>	<u>TSSTSKSTGG</u>
CpNPR1	IKI				
OsNPR1	IRPRR				
AtNPR1	KRSNRKLSHR	RR			

Fig. 7. Pileup (Genetics Computer Group, 1997) alignment of papaya (Cp), rice (Os, AP002537) and arabidopsis (At, U76707) NPR1 genes. Functional domains defined in arabidopsis and conserved in papaya and rice are underlined. The first domain is the POZ domain, followed by the ankyrin repeat region, followed by the NLS.

numerous PR genes. SA stimulates movement of NPR1 protein into the nucleus, and in the nucleus alters the redox state of TGA1 and possibly other TGA proteins. Binding of NPR1 to TGA factors results in binding of as-1-like elements by ASF-1 or SARP, thereby effecting SA induction of PR genes [13]. Structural domains required to carry out these functions include ankyrin repeats [6,35], a POZ domain [3], and an NLS. Ankyrin domains in other

proteins have been shown to mediate protein–protein interactions. Four arabidopsis mutations that abolished NPR1 function proved to be point mutations in the ankyrin repeats, and deletion of part of this region reduced binding to TGA transcription factors to base levels in a yeast two-hybrid assay [13,51,52]. POZ domains also mediate protein–protein interactions. Proof for such interactions has not been demonstrated for NPR1; however, a point

mutation in the POZ domain of the arabidopsis gene abolishes NPR1 function [6]. The arabidopsis gene contains three potential NLS; the second of these (aa residues 541–554) is necessary for NPR1 function and is required for nuclear localization of an NPR1-GFP fusion protein. Mutagenesis of five basic amino acids in this region resulted in localization of the fusion protein exclusively to the cytoplasm [20].

Papaya NPR1, like the arabidopsis gene has four exons and three introns, and the intron locations are identical. The deduced amino acid sequence is, overall, 67% similar to the arabidopsis sequence, and all three functional domains identified in arabidopsis are conserved in the papaya gene. Over the ankyrin repeat region, papaya NPR1 shows 73% similarity to arabidopsis. Over the POZ domain, papaya and arabidopsis share 85% similarity (28/33) to POZ consensus residues [3]. Of the five basic aa residues required for nuclear localization in arabidopsis, four are conserved in papaya, which is the same level of conservation seen in the rice [36] and tobacco [24] NPR1 genes. Conservation of these structural domains in papaya NPR1 suggests a similar function and role as those demonstrated in arabidopsis.

Papaya NPR1 is expressed in the absence of BTH, but BTH treatment slightly elevates mRNA levels, approximately  $1.7 \times$ . This low level of induction is similar to the pattern reported in arabidopsis, which is increased approximately twofold by INA or SA treatment [6]. While SA induction of NPR1 is small, it is very important for plant defense. Mutation of a WRKY box 'which binds an SA-inducible protein complex' in the arabidopsis NPR1 promoter compromised the ability of NPR1 to complement the *npr1* mutation [42].

*P. palmivora* does not trigger a hypersensitive response in papaya, and is able to systemically infect the plant, which eventually dies.  $\beta$ -1,3-Glucanase activity is not induced locally in roots of infected papaya. In leaves there is a twofold induction; however, this is considerably less than the induction observed from BTH treatment. For chitinase, infected roots do not show an increase in activity, while leaves show less than a twofold increase. Again, this increase is much less than observed following BTH treatment. These data are as expected for a compatible interaction: the plant responds with increased  $\beta$ -1,3-glucanase and chitinase activities, but at a much reduced level when compared to the response induced by BTH. Future work will determine how papaya defenses are mobilized in response to an avirulent pathogen.

Overall, the papaya SAR response is sufficiently similar to that observed in arabidopsis and tobacco that models based on those much-studied species are useful for structuring research in papaya. Exogenous treatment with BTH induces increased tolerance for a virulent pathogen, increases hydrolytic enzyme activities characteristic of SAR, and increased accumulation of a PR-1 mRNA. Papaya NPR1 conserves those structural domains that have been

shown to be required for function in arabidopsis and may play a similar role.

Infection with a virulent pathogen fails completely to induce some of the SAR elements induced by BTH and induces others to only a low degree.

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